DESCRIPTION

SPECIFIC SUBSTRATE FOR ENZYMES CLEAVING VON WILLEBRAND FACTOR AND METHOD FOR ASSAYING ACTIVITY THEREOF

Technical Filed of the Invention

The invention relates to a specific substrate for enzymes cleaving plasma proteins, in particular, von Willebrand factor, a method for assaying activity thereof, and a high throughput system for assaying enzymatic activity of cleaving von Willebrand factor.

Prior Art

Von Willebrand factor (hereinafter referred to as "VWF") is a plasma protein which plays a significant role in the blood coagulation. VWF is synthesized mainly in vascular endothelia and released into the bloodstream in multimeric forms of high molecular weights. Normally, VWF undergoes limited cleavage to forms of appropriate sizes by a von Willebrand factor cleaving enzyme (designated as ADAMTS-13 or VMF-CP, hereinafter referred to as "ADAMTS-13") in the plasma, and accordingly its activity of accelerating the coagulation is regulated. Significant decrease in the activity of ADAMTS-13 causes abnormal polymerization of VWF. As a result, thrombi which result particularly from excess aggregation of thrombocytes are formed, leading to a severe

systemic disease called thrombotic thrombocytopenic purpura (hereinafter referred to as "TTP"). TTP is broadly classified into congenital and acquired types.

Recently, ADAMTS-13 has been isolated, and its coding gene ADAMTS13 has been identified[1]. Mutation in this gene will be responsible for congenital TTP, whereas there is elucidated no mechanism of developing acquired TTP which accounts for the majority of TTP and is induced by pregnancy, side effects of drugs, and others.

TTP, which is a thrombotic systemic disease involving thrombocytopenia, will most likely lead to death, if left untreated. After the effectiveness of plasma exchange has become to be known, the fatality rate has been remarkably reduced. However, plasma exchanging once in every two to three weeks imposes heavy burdens on patients, and also risks such as infections are not negligible. Thus, there is a need for accurate and rapid measuring of ADAMTS-13 activity make an accurate judgment of the timing of plasma exchange, thereby to reduce the number of plasma exchanging and to increase therapeutic effects. In addition, accurate measuring of ADAMTS-13 activity is indispensable for the prediction of acquired TTP. Accurate measuring of ADAMTS-13 activity allows one to obtain clinical information that the activity is not decreased, that is, no sign of developing TTP appears, by periodically measuring the activity of ADAMTS-13

in the blood, for example, during taking drugs that have side effects of developing TTP, and during the pregnancy tending to induce TTP. It is also possible to make a definite diagnosis of patients with TTP, based on the decrease in ADAMTS-13 activity.

As a disease which displays clinical symptoms closely similar to TTP, there is known HUS (hemolytic uremic syndrome). In patients with HUS, however, the activity of ADAMTS-13 is in normal levels, which is contrast to the decrease or loss of ADAMTS-13 activity in TTP patients. Therefore, accurate measuring of the ADAMTS-13 activity of patients also allows one to make a discrimination between TTP and HUS.

ADAMTS-13 specifically cleaves the peptide bond between Try^{1605}-Met^{1606} of the VWF subunit[2]. It is not known that enzymes other than ADAMTS-13 specifically cleave this site. At present, there are known methods for measuring ADAMTS-13 activity, such as (i) combinations of electrophoresis and western blot of reaction solutions using purified VWF as the substrate[3], (ii) measurement of the ability of VWF to bind to collagen[4], (iii) quantitative determination using VWF site-specific monoclonal antibodies[5]. However, these methods have disadvantages, for example, of requiring much time and skill for their operation, lacking the quantitativeness, and having low sensitivity, and also lack

the simplicity and the capability of processing many samples, making it difficult for them to become used widely in the clinical field. In addition, it is said that it is impossible in the case of ADAMTS-13, due to the problem of the substrate specificity, to utilize chromogenic or fluorescent synthetic peptide substrates which are commonly used as high throughput systems for assaying of protease activities[6].

Problems to be Solved by the Invention

Although TTP caused by reduced activities of ADAMTS-13 is a very severe disease, as mentioned above, the current situation is that no method for accurate and rapid measuring of ADAMTS-13 activity has yet been established. Therefore, the present invention is intended to overcome the shortcomings of conventional methods for measuring ADAMTS-13 activity, thereby contributing to effective treatments of TTP, making a prediction of developing TTP, a definitive diagnosis of TTP, a discrimination between TTP and HUS, and others.

Means for Solving the Problems

In view of the above-described situation, the inventors have conducted extensive research, and have found that ADAMTS-13 specifically cleaves terminally cleaved, relatively

short partial amino acid sequences, or even mutant sequences thereof, of the mature VWF subunit, which contain the cleavage site that is between the 1605th amino acid tyrosine and the 1606th amino acid methionine of the amino acid sequence of wild-type human VWF depicted in SEQ ID NO: 1 (hereinafter, also expressed as Tyr^{1605}-Met^{1606}, and sometimes simply referred to as the "cleavage site"), resulting in successful measurement of ADAMTS-13 activity in a simple, specific, sensitive, and quantitative manner, thereby reaching the completion of the present invention.

Therefore, the present invention provides:

(1) a substrate polypeptide for ADAMTS-13, which begins at one of amino acids 764 to 1605 and ends at one of amino acids 1606 to 2813 of the amino acid sequence of wild-type human VWF depicted in SEQ ID NO: 1 in the Sequence Listing, wherein the polypeptide beginning at amino acid 764 and ending at amino acid 2813 of SEQ ID NO: 1 of the Sequence Listing is excluded;

(2) a substrate polypeptide for ADAMTS-13, which begins at one of amino acids 1459 to 1605 and ends at one of amino acids 1606 to 1668 of the amino acid sequence of wild-type human VWF depicted in SEQ ID NO: 1 in the Sequence Listing;

(3) a substrate polypeptide for ADAMTS-13, which begins at one of amino acids 1459 to 1600 and ends at one of amino acids 1611 to 1668 of the amino acid sequence of wild-type

human VWF depicted in SEQ ID NO: 1 in the Sequence Listing;

(4) a substrate polypeptide for ADAMTS-13, which begins at one of amino acids 1554 to 1600 and ends at one of amino acids 1660 to 1668 of the amino acid sequence of wild-type human VWF depicted in SEQ ID NO: 1 in the Sequence Listing;

(5) a substrate polypeptide for ADAMTS-13, which begins at amino acid 1587 and ends at amino acid 1668 of the amino acid sequence of wild-type human VWF depicted in SEQ ID NO: 1 in the Sequence Listing;

(6) a substrate polypeptide for ADAMTS-13, which begins at amino acid 1596 and ends at amino acid 1668 of the amino acid sequence of wild-type human VWF depicted in SEQ ID NO: 1 in the Sequence Listing;

(7) a mutant substrate polypeptide for ADAMTS-13, which has an amino acid sequence homology of at least 50% or higher to the substrate polypeptide for ADAMTS-13 according to any of (1) to (6) as described above;

(8) a mutant substrate polypeptide for ADAMTS-13, which has an amino acid sequence homology of at least 70% or higher to the substrate polypeptide for ADAMTS-13 according to any of (1) to (6) as described above;

(9) a mutant substrate polypeptide for ADAMTS-13, which has an amino acid sequence homology of at least 90% or higher to the substrate polypeptide for ADAMTS-13 according to any of (1) to (6) as described above;

(10) a mutant substrate polypeptide for ADAMTS-13, which is different from the substrate polypeptide for ADAMTS-13 according to any of (1) to (6) as described above, by one or more amino acid deletion, insertion, substitution, or addition (or combinations thereof) in the amino acid sequence of the substrate polypeptide for ADAMTS-13 according to any of (1) to (6) as described above;

(11) the substrate polypeptide or mutant substrate polypeptide for ADAMTS-13 according to any of (1) to (10) as described above, having a tag sequence attached at the N-termial and/or at the C-terminal;

(12) the substrate polypeptide or mutant substrate polypeptide for ADAMTS-13 according to (11) as described above, wherein the tag is selected the group consisting of proteins, peptides, coupling agents, radioactive labels, and chromophores;

(13) the substrate polypeptide or mutant substrate polypeptide for ADAMTS-13 according to (11) or (12) as described above, wherein the tag is for immobilization on a solid phase;

(14) the substrate polypeptide or substrate mutant polypeptide for ADAMTS-13 according to (13) as described above, which is immobilized on a solid phase;

(15) a method for measuring ADAMTS-13 activity in a subject, which comprises contacting a substrate polypeptide or mutant

substrate polypeptide for ADAMTS-13 according to any of (1) to (14) as described above, with plasma obtained from a normal subject, followed by analyzing resultant polypeptide fragments to make a control; and contacting said substrate polypeptide or mutant substrate polypeptide for ADAMTS-13 according to any of (1) to (14) as described above, with plasma obtained from the subject, followed by analyzing resultant polypeptide fragments in a similar way and making a comparison with the control;

(16) a high throughput method for measuring the activity of ADAMTS-13 in plasma from subjects, which comprises employing a substrate polypeptide or mutant substrate polypeptide for ADAMTS-13 according to any of (1) to (14) as described above;

(17) a diagnostic composition for *in vitro* test of the decrease or deficiency of ADAMTS-13 activity in a patient, comprising a substrate polypeptide or mutant substrate polypeptide for ADAMTS-13 according to any of (1) to (14) as described above;

(18) a kit for *in vitro* test of the decrease or deficiency of ADAMTS-13 activity in a patient, comprising as the essential component a substrate polypeptide or mutant substrate polypeptide for ADAMTS-13 according to any of (1) to (14) as described above; and

(19) use of a substrate polypeptide or mutant substrate polypeptide for ADAMTS-13 according to any of (1) to (14) as

described above, for producing the diagnostic composition according to (17) as described above or the kit according to (18) as described above.

Brief Description of the Drawings

Fig. 1 shows the results of reactions of GST-Asp^{1459}-Arg^{1668}-H, GST-Glu^{1554}-Arg^{1668}-H, GST-Asp^{1587}-Arg^{1668}-H, GST-Asp^{1596}-Arg^{1668}-H, and GST-Asp^{1596}-Arg^{1659}-H with normal plasma at 37°C for 2 hours, followed by separation on SDS-PAGE and western blot employing an anti-GST antibody as the primary antibody.

Fig. 2 shows the substrate specificity and reaction quantitativeness of GST-Asp^{1459}-Arg^{1668}-H. The reaction conditions were the same as in Fig. 1.

Detailed Description of the Invention

Wild-type human VWF is a polypeptide composed of 2813 amino acids in all, including its signal peptide and pro region. The amino acid sequence of wild-type human VWF is depicted in SEQ ID NO: 1 in the Sequence Listing. The mature subunit of wild-type human VWF, which is a segment excluding its signal peptide and pro region, is a polypeptide extending from amino acid 764 to amino acid 2813 of SEQ ID NO: 1 in the Sequence Listing. The numbering of the amino acids is as follows: the initial methionine at the amino (N) end of wild-type human VWF is set to be 1 (amino acid 1), and the amino

acids are numbered consecutively in the direction toward the carboxyl (C) end (see, SEQ ID NO: 1 in the Sequence Listing). In this specification, the 1459th amino acid from the N-termial of SEQ ID NO: 1 in the Sequence Listing is sometimes expressed as amino acid 1459, for example. Further, the 1459th amino acid from the N-termial of SEQ ID NO: 1 in the Sequence Listing is aspartic acid (Asp), and in some cases, is expressed as Asp^{1459}. Furthermore, for example, the polypeptide extending from amino acid 1459 (Asp) to amino acid 1668 (Arg) is, in some cases, expressed as Asp^{1459}-Arg^{1668}.

The amino acid sequence of "wild-type" VWF means the amino acid sequence of human VWF which is not mutated. In this specification, unless specified to be "mutant," amino acid sequences are not intended to be "mutant," even in the absence of the expression of "wild-type."

Therefore, in this specification, if a partial amino acid sequence of the human mature VWF subunit has the same sequence as that of the native human mature VWF subunit corresponding to that segment, the partial sequence is "wild-type," and if a partial amino acid sequence has a different sequence, the partial sequence is "mutant."

As used herein, the VWF is of human origins, unless specified to be from non-human. VWFs originating from non-human organisms are included in "mutant" versions, in this

specification.

A "polypeptide" as used herein refers to a peptide having two or more amino acid residues. Further, the terms "polypeptide" and "protein" are used synonymously in this specification.

In this specification, amino acids are expressed by the conventional three-letters.

The present invention, in one embodiment, provides a substrate polypeptide for ADAMTS-13, which begins at one of amino acids 764 to 1605 and ends at one of amino acids 1606 to 2813 of the amino acid sequence of wild-type human VWF depicted in SEQ ID NO: 1 in the Sequence Listing, wherein the polypeptide beginning at amino acid 764 and ending at amino acid 2813 of SEQ ID NO: 1 of the Sequence Listing is excluded.

The substrate polypeptide for ADAMTS-13 according to the present invention has a partial amino acid sequence of the human mature VWF subunit comprising the cleavage site Tyr^{1605}-Met^{1606} as the essential component. Therefore, the full-length wild-type human mature VWF subunit is excluded which begins at amino acid 764 and ends at amino acid 2813.

The region from amino acid 1459 (Asp) to amino acid 1668 (Arg) of the amino acid sequence of wild-type human VWF depicted in SEQ ID NO: 1 in the Sequence Listing, which contains no Cys residue, does not cause multimerization due

to the formation of disulfide linkage and does not get rise to problems in the specificity for ADAMTS-13, quantitativeness of measuring ADAMTS-13 activity, reproducibility, handling, and others. Therefore, the present invention, in a preferable embodiment, provides a substrate polypeptide for ADAMTS-13, which begins at one of amino acids 1459 to 1605 and ends at one of amino acids 1606 to 1668 of the amino acid sequence of wild-type human VWF depicted in SEQ ID NO: 1 in the Sequence Listing.

Short polypeptides consisting of up to four amino acids in front of and behind the cleavage site, respectively, are not much specific for ADAMTS-13, as a substrate. Therefore, the present invention, in a more preferable embodiment, provides a substrate polypeptide for ADAMTS-13, which begins at one of amino acids 1459 to 1600 and ends at one of amino acids 1611 to 1668 of the amino acid sequence of wild-type human VWF indicated in SEQ ID NO: 1 in the Sequence Listing. The substrate polypeptide for ADAMTS-13 of this embodiment, which contains no Cys residue as mentioned above, does not cause multimerization due to the formation of disulfide linkage and does not get rise to problems in the specificity for ADAMTS-13, quantitativeness of measuring ADAMTS-13 activity, reproducibility, handling, and others. In addition, the polypeptide for ADAMTS-13 of this embodiment is of a small size which is sufficiently suitable for producing

it by recombinant methods, and has high specificity for ADAMTS-13.

The present invention, in a further preferable embodiment, provides a substrate polypeptide for ADAMTS-13, which begins at one of amino acids 1554 to 1600 and ends at one of amino acids 1660 to 1668 of the amino acid sequence of wild-type human VWF depicted in SEQ ID NO: 1 in the Sequence Listing. As mentioned above, the substrate polypeptide for ADAMTS-13 of this embodiment, which contains no Cys residue, does not cause multimerization due to the formation of disulfide linkage and does not get rise to problems in the specificity for ADAMTS-13, quantitativeness of measuring ADAMTS-13 activity, reproducibility, handling, and others. The substrate polypeptide for ADAMTS-13 of this embodiment has a smaller size than that of the polypeptide of the above-described embodiment, and thus is particularly suitable for producing it by recombinant methods. In addition, the substrate polypeptide for ADAMTS-13 of this embodiment has higher specificity for ADAMTS-13 than that of the polypeptide of the above-described embodiment (see, the section of Examples).

The present invention provides, as a particularly preferable specific example, a substrate polypeptide for ADAMTS-13, which begins at amino acid 1587 and ends at amino acid 1668 of the amino acid sequence of wild-type human VWF

depicted in SEQ ID NO: 1 in the Sequence Listing, and a substrate polypeptide for ADAMTS-13, which begins at amino acid 1596 and ends at amino acid 1668 of the amino acid sequence of wild-type human VWF depicted in SEQ ID NO: 1 in the Sequence Listing.

These substrate polypeptides for ADAMTS-13 of the present invention are cleaved between Tyr^{1605}-Met^{1606} by ADAMTS-13.

In Addition, the present invention, in a further embodiment, provides a mutant substrate polypeptide for ADAMTS-13, which has an amino acid homology of at least 50% or higher, preferably at least 70% or higher, and more preferably at least 90% or higher, to the substrate polypeptide for ADAMTS-13 according to any of the above-described embodiments.

Preferably, the mutant substrate polypeptide for ADAMTS-13 has the cleavage site Tyr^{1605}-Met^{1606} contained therein. However, as long as the mutant substrate polypeptide for ADAMTS-13 retains specificity for ADAMTS-13, the two amino acids of the cleavage site may be different from the above-described amino acids (Tyr^{1605}, Met^{1606}), and such mutant substrate polypeptides for ADAMTS-13 are also encompassed within the present invention.

The "homology" of an amino acid sequence refers to the degree at which two or more amino acid sequences under

comparison have the identical or a similar amino acid sequence. For the mutant substrate polypeptides for ADAMTS-13 according to the present invention, amino acid sequences having 100% homology to the wild type are excluded.

Further preferably, a mutant substrate polypeptide for ADAMTS-13 according to the present invention is a mutant substrate polypeptide for ADAMTS-13 which is different from the above-described substrate polypeptide for ADAMTS-13, by one or more amino acid deletion, insertion, substitution, or addition (or combinations thereof) in the amino acid sequence of the above-described substrate polypeptide for ADAMTS-13.

Mutant amino acid sequences can be any sequence, if they are sequences as described, and preferably can be, for example, sequences having one or more amino acid deletion, insertion, substitution, or addition (or combination thereof) in the wild-type amino acid sequence, or sequences having a modified side chain(s) of one or more amino acids of the wild-type amino acid sequence (for example, synthetic, non-naturally occurring amino acids), or combinations of these alterations.

These alterations can result from spontaneous mutation or artificial mutagenesis. Artificial mutagenesis is well known in the art and includes, for example, site-directed mutagenesis employing recombinant procedures, synthesis of mutant polypeptides by chemical processes, such as solid-

phase synthesis and liquid-phase synthesis, or chemical modification of amino acid residues, details of each of which are well known to those skilled in the art. Additionally, such mutation and/or modification can be made at any position.

Examples of modifying amino acids are, for example, acetylation, acylation, amidation, addition of sugar chains, addition of nucleotides or nucleotide derivatives, addition of lipids or lipid derivatives, cyclization, formation of disulfide linkage, demethylation, formation of cross-linking, formation of cystine, formation of pyroglutamic acid, formylation, hydroxylation, halogenation, methylation, oxidation of side chains, treatments with proteolytic enzymes, phosphorylation, sulfation, racemization, and others, which are well-known in the art.

Especially when a substrate polypeptide for ADAMTS-13 according to the present invention is produced in a eukaryotic cell expression system, it is highly likely that a sugar chain is added at a serine or threonine residue(s) of the polypeptide. Substrate polypeptides for ADAMTS-13 which are expressed in this way in eukaryotic cells and undergo the addition of a sugar chain are also included within the present invention.

The following describes methods for producing substrate polypeptides or mutant substrate polypeptides for ADAMTS-13

of the present invention. Although the explanation which follows is made on methods for producing substrate polypeptides for ADAMTS-13 of the present invention, it will be clear to those skilled in the art that the explanation which follows is also applicable to methods for producing mutant substrate polypeptides for ADAMTS-13.

When chemical synthesis is carried out, solid- or liquid-phase peptide synthesis is commonly used. For example, solid-phase peptide synthesizers can be employed. When modification of amino acid residues is required, modified amino acids can be introduced into a synthesizer as appropriate. It is also well known to introduce a protecting group into a sensitive residue during the synthesis. In addition, modifications may be performed after the amino acid sequence is obtained. Needless to say, these and other chemical synthesis procedures are well known in the art, and those skilled in the art can select an appropriate procedure to synthesize intended polypeptides.

Alternatively, it is also possible to produce a polypeptide of the present invention by digesting a polypeptide containing the polypeptide of the present invention with an appropriate protease and/or peptidase. For example, a VWF fraction may be separated from plasma and subjected to the reaction with a protease and/or peptidase having a specific cleavage site.

Methods of isolating and purifying resulting polypeptides are also well known in the art, such as chromatography of various types, salting out, electrophoresis, ultrafiltration, and others.

Also, it is possible to produce substrate polypeptides for ADAMTS-13 of the present invention by recombinant procedures. Production of polypeptides by recombinant procedures can be carried out by methods well known to those skilled in the art, such as methods described by Sambrook et al. in Molecular Cloning, 2nd Ed., Cold Spring Harbor Laboratory Press (1989), while typical procedures are described below.

First, DNA coding for a polypeptide of the present invention is cloned. Means for cloning DNA include, for example, methods in which synthetic DNA primers having a partial base sequence of the polypeptide of the present invention are employed so as to allow one to carry out amplification by methods well known in the art, such as PCR procedures. The cloned DNA is ligated into an appropriate expression vector, which in turn is introduced into an appropriate host cell to transform the host cell, and the transformed host cell is cultured, thereby allowing one to obtain the expressed polypeptide. It is preferable that when ligating the cloned DNA into an appropriate expression vector, the DNA is ligated downstream of an appropriate

promoter to facilitate its expression for obtaining much amounts of the polypeptide. The nucleotide sequence of human VWF has been deposited on a database, for example, as the GenBank Accession No. NM_000552 and is available.

Expression vectors include plasmids from *E. coli* (for example, pBR322, pBR325, pUC12, pUC13, and others), plasmids from *Bacillus subtilis* (for example, pUB10, pTP5, pC194, and others), plasmids from yeast (for example, pSH19, pSH15, and others), bacteriophages (for example, lambda phage and others), baculoviruses, animal viruses (for example, retrovirus, vaccinia virus, and others), or pA1-11, pXXT1, pRc, pcDNAI, and the like. These and other vectors are well known to those skilled in the art, and many vectors are commercially available (for example, pGEX-6P-1, which is commercially available from Amersham-Bioscience, is a vector allowing for expression of fusion proteins with a tag protein, glutathione-S-transferase).

Host cells include bacterial cells, such as *E. coli* (for example, strains K12, HB101, JM103, JA221, C600, BL21, and others), *Bacillus subtilis*, and genera *Streptococcus*, *Staphylococcus*, *Enterococcus*; fungus cells, such as yeast cells and *Asperguillus* cells; insect cells, such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells, such as CHO, COS, HeLa, C127, 3T3, BHK, 293 cells; and plant cells.

Any promoters can be used, as long as they are suitable

for host cells which are employed for the expression of DNA coding an intended polypeptide; for *E. coli* hosts, trp, lac, recA promoters, for example, are employed; for *Bacillus subtilis* hosts, SPO1, SPO2, penP promoters, for example; for yeast hosts, PHO5 and PGK promoters, for example; for insect hosts, P10 and polyhedron promoters, for example; for animal cell hosts, SV40 early, SR-alpha, CMV promoters, for example.

Expression vectors may further contain, if desired, enhancers, splicing signals, poly A addition signals, selectable markers (antibiotic-resistance genes such as genes resistant to methotrexate, ampicillin, and neomycin, dihydrofolate reductase gene, and others).

Transformation of host cells can be performed according to methods described in many texts, including the above-described text by Sambrook et al., such as calcium phosphate protocols, methods employing DEAE-dextran, microinjection, electroporation, and virus infection.

When culturing the transformants, liquid media are suitable as a medium which can be used, and preferable medium compositions and culture conditions for respective host types are well known in the art, and can be selected by those skilled in the art.

It is possible to incorporate an appropriate secretion signal into a polypeptide to be expressed, in order to allow the translated polypeptide to be secreted into the

endoplasmic reticulum lumen, periplasmic space, or extracellular environment. Such a signal may be a signal native or heterologous to the polypeptide.

Expressed recombinant polypeptides can be recovered and purified from recombinant cell cultures by well-known methods, including, for example, ammonium sulfate or ethanol precipitation, precipitation with organic solvents, electrophoresis, ultrafiltration, anion- or cation-exchage chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, and lectin chromatography, and others. It is preferable to employ high performance liquid chromatography for purification. In the case where the polypeptide is denatured during the isolation and/or purification, for example, where the polypeptide is produced as inclusion body within the bacterial cell, well-known techniques for the regeneration of polypeptides, such as urea treatment, can be utilized to make the denatured polypeptide have the active conformation again.

The following describes the determination of the activity using a substrate polypeptide or mutant polypeptide for ADAMTS-13 of the present invention, and diagnostic compositions and kits comprising such a polypeptide. Although the explanation which follows is made with respect to substrate polypeptides for ADAMTS-13, it will be clear to

those skilled in the art that the explanation which follows is also applicable to mutant polypeptides for ADAMTS-13.

The activity of ADAMTS-13 in a subject can be measured using a substrate polypeptide for ADAMTS-13 of the present invention, for example, in the following procedures: Under appropriate reaction conditions, a substrate polypeptide for ADAMTS-13 of the present invention is contacted with plasma obtained from a normal subject, and resultant polypeptide fragments are analyzed, for example, on SDS-polyacrylamide gel (hereinafter, referred to as "SDS-PAGE") to make a control, and the substrate polypeptide for ADAMTS-13 of the present invention is contacted with plasma obtained from a subject and subjected to SDS-PAGE in a similar way, followed by staining of proteins by Coomassie Blue or silver staining or the like and analyzing the products to compare the band position, density, and the like with the control. Alternatively, it may be possible to carry out western blotting following the SDS-PAGE. The reaction solution preferably contains divalent metal ions, such as Ba^{2+}, which are an ADAMTS-13 activator, and in addition, a buffer solution whose pH corresponds to the optimal pH of 8 to 9 of ADAMTS-13.

Thus, the present invention relates to a method for measuring the activity of ADAMTS-13 in a subject plasma, which comprises contacting a substrate polypeptide for

ADAMTS-13 of the present invention with plasma obtained from a subject and analyzing the product, as described above.

The present invention also relates to a diagnostic composition for *in vitro* test of the decrease or deficiency of ADAMTS-13 activity in a subject, and therefore the presence of TTP or the predisposition to TTP, or for making a definitive diagnosis of TTP and a discrimination between TTP and HUS, wherein the composition comprises a substrate polypeptide for ADAMTS-13 of the present invention. The present invention further relates to a kit for *in vitro* test of the decrease or deficiency of ADAMTS-13 activity in a subject, and therefore the presence of TTP or the predisposition to TTP, or for making a definitive diagnosis of TTP and a discrimination between TTP and HUS, wherein the kit comprises as the essential component a substrate polypeptide for ADAMTS-13 of the present invention. The kit usually has its instructions accompanied therewith.

A substrate polypeptide or mutant polypeptide for ADAMTS-13 of the present invention may have a tag sequence attached at the N-termial and/or at the C-terminal. Although the explanation which follows is made with respect to substrate polypeptides for ADAMTS-13, it will be clear to those skilled in the art that a tag also can be attached to mutant polypeptides for ADAMTS-13 and used in a similar way. The tag sequence may be any one, and preference is given to

tag sequences which facilitate, for example, detection, quantification, and separation of cleaved products by ADAMTS-13. Also, the tag sequence may be for immobilizing a substrate polypeptide for ADAMTS-13 of the present invention onto a solid phase. The present invention also encompasses substrate polypeptides for ADAMTS-13 which are immobilized onto a solid phase using such tag sequences. The tag sequences include proteins (for example, glutathione transferase (hereinafter, referred to as "GST"), luciferase, beta-galactosidase, and others), peptides (for example, His tag and others), coupling agents (carbodiimide reagents and others), various kinds of labels (for example, radioactive labels, chromophores, enzymes, and others), and those skilled in the art can select the type of tags according to the purpose. Methods for attaching of tag are well known to those skilled in the art.

For example, as detailed in Example 1, DNA coding for a substrate polypeptide for ADAMTS-13 of the present invention may be inserted into the *E. coli* expression vector pGEX-6P-1 to obtain a fusion protein having, as a tag, GST fused at the N-termial of the substrate polypeptide for ADAMTS-13 of the present invention. In this case, the fusion protein can be purified by affinity chromatography employing a glutathione sepharose column. For example, when macromolecules such as GST protein are fused, two fragments whose molecular weights

are significantly different can be analyzed after the reaction; for example, the reaction products are separated, for example, on SDS-PAGE, whereby their analysis will become easier. In this case, when an anti-GST antibody is available, the antibody can be used for western blotting.

In addition, for example, luciferase or galactosidase as a tag sequence may be fused at the C-terminal of a substrate polypeptide for ADAMTS-13 of the present invention and GST at the N-termial. In this case, the fusion protein can be trapped, for example, on glutathione beads, and the tagged product which is released after the cleavage with ADAMTS-13 can be quantified by well known methods for measuring the activity of luciferase or galactosidase, thereby determining the ADAMTS-13 activity.

Further, well-known His tags, anti-Myc tags, and others can be also used as tag sequences. For example, a His tag is added at the N-termial of a substrate polypeptide for ADAMTS-13 of the present invention for immobilization onto a solid phase and a horseradish peroxides (HRP) labeled anti-Myc tag at the C-terminal. After the reaction with ADAMTS-13, the HRP released into the liquid phase can be determined colorimeterically by well-known methods, thereby determining the ADAMTS-13 activity.

In addition, a specific embodiment as described below is also considered to be an embodiment in which a tag is

added to a substrate polypeptide for ADAMTS-13 of the present invention. Accordingly, a known protein is selected whose activity-measuring method has been already established, and the amino acid sequence of a substrate polypeptide for ADAMTS-13 of the present invention is inserted into the amino acid sequence of the known protein so as to retain the activity of the known protein, thereby to obtain a fusion protein. This fusion protein has been adapted such that when the fusion protein is reacted with plasma obtained from a subject and cleaved at the cleavage site by the ADAMTS-13 activity in plasma, the activity of the initial known protein becomes lost. This type of fusion protein also can be used to determine the activity of ADAMTS-13 in plasma by measuring the degree of the loss of the activity of the fusion protein.

It is also possible to make a substrate polypeptide or mutant substrate polypeptide for ADAMTS-13 of the present invention suitable for high throughput measuring of ADAMTS-13 activity, for example, by addition of a tag allowing or facilitating detection, or by immobilization onto a solid phase. Therefore, the present invention relates to a method, preferably a high throughput method, for measuring the activity of ADAMTS-13 in plasma from subjects, wherein the method is characterized by employing preferably a substrate polypeptide or mutant substrate polypeptide for ADAMTS-13 of the present invention to which a tag is added. The present

invention also relates to a composition or a kit for measuring the activity of ADAMTS-13 in plasma, wherein the composition or the kit comprises a substrate polypeptide or mutant substrate polypeptide for ADAMTS-13 of the present invention to which a tag is added.

In further embodiments, the present invention relates to use of a substrate polypeptide or mutant substrate polypeptide for ADAMTS-13 as described above, for producing the diagnostic composition or the kit as described above.

Examples

A. Production of substrate peptides for ADAMTS-13

As mentioned above, ADAMTS-13 specifically cleaves the peptide bond between Tyr^{1605}-Met^{1606} of VWF. In fact, however, VWF undergoes the aggregation of many mature subunits, resulting in the formation of huge molecules, and thus causes problems in the quantitativeness, reproducibility, operationality, and the like, when as in conventional measuring methods, native VWF is used as the substrate. In the present invention, measuring of the enzymatic activity of ADAMTS-13 utilizes, as the substrate, partial sequences of the mature VWF subunit which contain sequences around its cleavage site, thereby leading to the solution of these problems.

The partial sequences should in general have a certain

length in order to retain the substrate specificity for ADAMTS-13, whereas it would be better that they have a small size in order to make them suitable for production through recombinant expression by *E. coli*. When used as the substrate, the partial sequences should represent regions containing no cysteine residue, since the presence of a cysteine residue having an SH group in a polypeptide may cause multimerization, resulting in problems in the quantitativeness, handling, reproducibility, and the like. We selected the polypeptide Asp^{1459}-Arg^{1668} as a polypeptide meeting these requirements. That is, this region is the longest segment that contains the cleavage site and carries no cysteine residue.

RT-PCR was carried out using, as the template, RNA extracted from commercially available human umbilical veiN-termialothelial cells, to obtain cDNA coding for the Asp^{1459}-Arg^{1668} region of the VWF subunit (SEQ ID NO: 2). The sense-direction primer used was 5′-cgggatccGACCTTGCCCCTGAAGCCCCTC-3′ (SEQ ID NO: 7) and the antisense-direction primer was 5′-ggaattcTCAGTGATGGTGATGGTGATGCCTCTGCAGCACCAGGTCAGGA-3′ (SEQ ID NO: 8) (the portions of lower case letters represent restriction enzyme recognition sites added for subcloning). The antisense-direction primer has a 6xHis tag sequence added thereto. The PCR product was digested with BamHI and EcoRI and then inserted into the *E. coli* expression vector pGEX-6P-

1 (Amersham-Bioscience) which had been digested with the same enzymes, BamHI and EcoRI, so as to express a fusion protein which has glutathione-S-transferase (GST) attached at the N-termial and the 6xHis tag sequence attached at the C-terminal of the Asp^{1459}-Arg^{1668} region of the VWF subunit (hereinafter, designated as GST-Asp^{1459}-Arg^{1668}-H). The resulting expression vector was introduced into *E. coli* strain BL21, which in turn was subjected to transient expression by IPTG induction, followed by purification through nickel-affinity chromatography and glutathione-affinity chromatography to obtain the fusion protein GST-Asp^{1459}-Arg^{1668}-H.

Smaller polypeptides than the above-described polypeptide are more suitable for production by recombinant methods using *E. coli* or others. In order to obtain cDNAs coding the Glu^{1554}-Arg^{1668} (SEQ ID NO: 3), Asp^{1587}-Arg^{1668} (SEQ ID NO: 4), Asp^{1596}-Arg^{1668} (SEQ ID NO: 5), and Asp^{1596}-Arg^{1659} (SEQ ID NO: 6) regions, three sense-direction primers 5′-cgggatccGAGGCACAGTCCAAAGGGGACA-3′ (SEQ ID NO: 9), 5′-cgggatccGACCACAGCTTCTTGGTCAGCC-3′ (SEQ ID NO: 10), and 5′-cgggatccGACCGGGAGCAGGCGCCCAACC-3′ (SEQ ID NO: 11), and one antisense-direction primer 5′-cggaattcTCAGTGATGGTGATGGTGATGTCGGGGGAGCGTCTCAAAGTCC-3′ (SEQ ID No: 12) were employed. They were combined and processed in a similar way to produce plasmids allowing the expression of four fusion proteins, GST-Glu^{1554}-Arg^{1668}-H, GST-Asp^{1587}-

Arg^{1668}-H, GST-Asp^{1596}-Arg^{1668}-H, and GST-Asp^{1596}-Arg^{1659}-H. Each of these expression vectors was introduced into *E. coli* strain BL21, which in turn was subjected to transient expression by IPTG induction, followed by purification through nickel-affinity chromatography and glutathione-affinity chromatography to obtain each of the fusion proteins.

When the five fusion proteins thus produced, GST-Asp^{1459}-Arg^{1668}-H, GST-Glu^{1554}-Arg^{1668}-H, GST-Asp^{1587}-Arg^{1668}-H, GST-Asp^{1596}-Arg^{1668}-H, and GST-Asp^{1596}-Arg^{1659}-H, are specifically cleaved by ADAMTS-13, that is, when the site corresponding to the site between Tyr^{1605}-Met^{1606} of the VWF subunit is cleaved, these fusion proteins will be separated into two fragments of 43.1 kDa (including the GST portion) and 7.7 kDa (including the His6 tag sequence portion), of 32.7 kDa and 7.7 kDa, of 29.0 kDa and 7.7 kDa, of 28.0 kDa and 7.7 kDa, and of 28.0 kDa and 6.7 kDa, respectively.

B. Reaction of substrate polypeptides for ADAMTS-13 with plasma ADAMTS-13

These fusion proteins were subjected to reactions with 0.25 μL of normal plasma at 37°C for zero and two hours. The total reaction volume was 20 μL, containing 25 mM Tris (pH 8.0), 10 mM $BaCl_2$, 4 mM glutathione, 1 mM APMSF. The reaction solutions were subjected to SDS-polyacrylamide gel electrophoresis for separation of the product, followed by

western blotting using an anti-GST antibody as the primary antibody. The results are shown in Fig. 1.

In the case of the two-hour reaction, the expected fragment (indicated by the arrowheads in the figure) was clearly yielded for GST-Asp^{1587}-Arg^{1668}-H and GST-Asp^{1596}-Arg^{1668}-H, while a very faint band was produced at the expected position, also for GST-Glu^{1554}-Arg^{1668}-H having a longer region. It proved that GST-Asp^{1459}-Arg^{1668}-H having a further longer region and GST-Asp^{1596}-Arg^{1659}-H having a shorter region did not give the fragment or was difficult to give the fragment. These results suggested that GST-Asp^{1587}-Arg^{1668}-H and GST-Asp^{1596}-Arg^{1668}-H be suitable as a substrate for ADAMTS-13.

C. Substrate specificity and reaction quantitativeness of substrate polypeptides for ADAMTS-13

In order to examine the specificity of GST-Asp^{1596}-Arg^{1668}-H as the substrate, among the particularly preferable substrate polypeptides for ADAMTS-13 obtained in Section B, it was reacted with plasma samples from TTP-patient family members. The reaction conditions and detection method were the same as described above. The results are shown in Fig. 2.

When each of plasma samples from two patients was reacted with GST-Asp^{1596}-Arg^{1668}-H, there was not detected the fragment which is yielded by the reaction with normal plasma

(indicated by the arrowhead in the figure). On the other hand, for plasma samples from mother and elder sister of family A, and from father and mother of family B which were found to have about one-half of the activity of ADAMTS-13 in normal plasma by another method, the fragment was yielded at smaller amounts than with the normal plasma. For a plasma sample from father of family A which was found to have an even lower activity, the fragment was yielded only at a further reduced amount. These results suggest that GST-Asp^{1596}-Arg^{1668}-H is a specific artificial substrate which is cleaved quantitatively by ADAMTS-13 in plasma and is not cleaved by other enzymes.

Industrial Applicability

The substrates polypeptides for measuring ADAMTS-13 activity of the present invention are small polypeptides which retain the substrate specificity and at the same time, are suitable for their production by recombinant expression by *E. coli*. In addition, the polypeptides contain no cysteine residue having a SH group, and thus can avoid the problem of their multimerization and also get rise to few problems relating to the quantitativeness, handling, reproducibility, and others. Therefore, the substrates polypeptides for measuring ADAMTS-13 activity of the present invention are capable of performing simple, specific,

quantitative, reproducible, and sensitive measurement of ADAMTS-13 activity. The substrates polypeptides for measuring ADAMTS-13 activity of the present invention are also suitable for multi-sample processing. For example, multi-sample processing can be carried out, for example, by immobilizing or labeling a substrate polypeptide for measuring ADAMTS-13 activity of the present invention.

The present invention enables one to make an effective treatment of TTP and a prediction of the onset of TTP. Specifically, the present invention allow one to obtain clinical information that the activity is not decreased, that is, no sign of developing TTP appears, by periodically measuring the activity of ADAMTS-13 in the blood, for example, during taking drugs that have side effects of developing TTP, and during the pregnancy tending to induce TTP. In addition, the present invention provides as a powerful tool for revealing the relationship between the ADAMTS-13 activity and a variety of diseases by epidemiological research. The present invention also allows one to make a rapid, definitive diagnosis of patients suffering from TTP. Further, the present invention allows one to make a precise measurement of the ADAMTS-13 activity of patients, thereby making a discrimination between TTP and HUS.

Free text in the Sequence Listing

SEQ ID NO: 1 depicts the amino acid sequence of wild-type human VWF.

SEQ ID NO: 2 depicts the amino acid sequence of Asp^{1459}-Arg^{1668}, a substrate polypeptide for ADAMTS-13 of the present invention.

SEQ ID NO: 3 depicts the amino acid sequence of Glu^{1554}-Arg^{1668}, a substrate polypeptide for ADAMTS-13 of the present invention.

SEQ ID NO: 4 depicts the amino acid sequence of Asp^{1587}-Arg^{1668}, a substrate polypeptide for ADAMTS-13 of the present invention

SEQ ID NO: 5 depicts the amino acid sequence of Asp^{1596}-Arg^{1668}, a substrate polypeptide for ADAMTS-13 of the present invention.

SEQ ID NO: 6 depicts the amino acid sequence of Asp^{1596}-Arg^{1659}, a substrate polypeptide for ADAMTS-13 of the present invention.

SEQ ID NO: 7 depicts the nucleotide sequence of the sense primer used for producing Asp^{1459}-Arg^{1668}, a substrate polypeptide for ADAMTS-13 of the present invention.

SEQ ID NO: 8 depicts the nucleotide sequence of the anti-sense primer used for producing Asp^{1459}-Arg^{1668}, a substrate polypeptide for ADAMTS-13 of the present invention.

SEQ ID NO: 9 depicts the nucleotide sequence of the sense

primer used for producing Glu^{1554}-Arg^{1668}, Asp^{1587}-Arg^{1668}, Asp^{1596}-Arg^{1668}, and Asp^{1596}-Arg^{1659}, substrate polypeptides for ADAMTS-13 of the present invention.

SEQ ID NO: 10 depicts the nucleotide sequence of the sense primer used for producing Glu^{1554}-Arg^{1668}, Asp^{1587}-Arg^{1668}, Asp^{1596}-Arg^{1668}, and Asp^{1596}-Arg^{1659}, substrate polypeptides for ADAMTS-13 of the present invention.

SEQ ID NO: 11 depicts the nucleotide sequence of the sense primer used for producing Glu^{1554}-Arg^{1668}, Asp^{1587}-Arg^{1668}, Asp^{1596}-Arg^{1668}, and Asp^{1596}-Arg^{1659}, substrate polypeptides for ADAMTS-13 of the present invention.

SEQ ID NO: 12 indicates the nucleotide sequence of the anti-sense primer used for producing Glu^{1554}-Arg^{1668}, Asp^{1587}-Arg^{1668}, Asp^{1596}-Arg^{1668}, and Asp^{1596}-Arg^{1659}, substrate polypeptides for ADAMTS-13 of the present invention.

References:

1) Levy GG et al., Nature, 2001, 413:488-494.

2) Dent JA et al., Proc Natl Acad Sci USA, 1990, 87:6306-6310.

3) Furlan M et al., Blood, 1996, 87:4223-4234.

4) Gerritsen H et al., Thromb Haemost, 1999, 82:1386-1389.

5) Obert B et al., Thromb Haemost, 1999, 82:1382-1385.

6) Furlan M et al., Seminars in Thromb and Hemost, 2002, 28(2):167-171.